The Effects of Light at Night on Immune Organ Clock Gene Expression in Siberian Hamsters (*Phodopus sungorus*)

Honors Research Thesis

Presented in Partial Fulfillment of the Requirements for graduation "with Honors Research Distinction in Neuroscience" in the undergraduate colleges of the Ohio State University

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April 2015

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Abstract

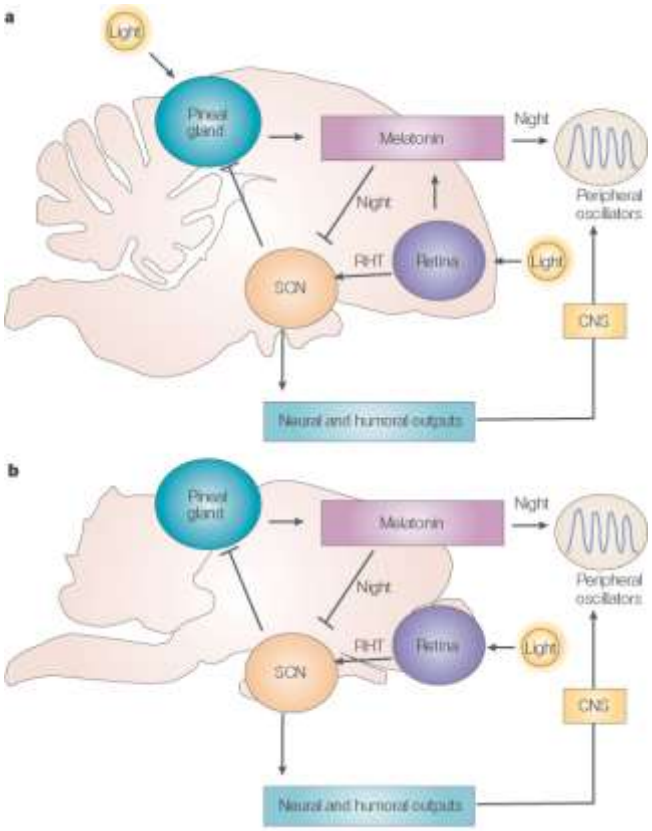
Exposure to light at night (LAN; i.e, light pollution) can interfere with seasonal changes in physiology and behavior in photoperiodic rodents. For example, non-tropical rodents turn off reproduction and bolster immune function in response to short days to improve their odds of winter survival. For Siberian hamsters in particular, dim light at night alters the development of the short-day (winter-like) phenotype and blocks enhanced immune function. The immune and circadian systems are tightly linked. Clock gene expression and their influence on inflammation in peripheral tissues are not well documented. The goal of this study was to explore the underlying gene responses in Siberian hamsters exposed to dim light at night by assaying clock genes and cytokine production in the lymph nodes and spleen. In a full factorial design, Siberian hamsters were exposed to either LD (long day) photoperiods, SD (short day) photoperiods, LD dim light at night (long day dim) photoperiods, and SD dim light at night (short day dim) photoperiods over a period of ten weeks. After extracting RNA from various peripheral organs, lymph nodes, and the spleen, quantitative PCR (qPCR) was performed to analyze gene expression. Dim light at night abolished the short-day body weight, organ mass, and pelage responses in Siberian hamsters. qPCR data and result discussion are forthcoming. Our results may suggest that LAN alters clock gene expression and cytokine production, as well as compromised immune function.

Introduction

Photoperiodism is the biological ability to measure day length (photoperiod) (Ko and Takahashi, 2006). Photoperiod can be used by animals as a reliable environmental cue to ascertain when certain biological functions should occur. Animals can also determine whether day lengths are increasing or decreasing in order to approximate the time of year (Weil et al., 2014). Certain physiological changes, such as fluctuating sleep patterns, body mass, pelage, and immune function, are directly influenced by changing photoperiods and help the animal acclimate to seasonal adaptations with proper timing. These seasonal changes, which are centered around day length, aid in reproduction and survival.

Immune function also changes with photoperiod. During winter-like, short day lengths, the immune system and its defenses in certain photoperiodic animals, such as Siberian hamsters, is increased. Short day lengths reroute energy from reproduction and growth to bolster immune function during winter (Nelson, 2004). Short day lengths are accompanied by increased melatonin secretion from the pineal gland, which works as an immunomodulator in many species, including humans (Nelson, 2004). For example, it acts on lymphocytes to enhance the division of immune cells in rodents and has been shown to increase the number of white blood cells in chickens *in vitro* (Nelson, 2004). In addition to melatonin secretion, there are specific responses to the environment, such as peripheral cytokine release, that also overlap with endocrine and nervous system functions, as well (Weil et al., 2014). This release is not only a sign of infection, but also of the effects from seasonal changes of the neuroendocrine-immune system as mentioned above. Signaling of

cytokines to the CNS affects many aspects of brain function, including pituitary development, hormone release from the anterior pituitary, and neuronal excitability (Weil et al., 2014). These cytokines can also affect the peripheral immune system through hypothalamic-pituitary-adrenal (HPA) axis activation and the secretion of the hormone melatonin from the pineal gland. Melatonin is important in the regulation of circadian rhythms, and relays day length signals from lighting cues in the environment (Weil et al., 2014). It translates photoperiodic information, and changes in day length, to peripheral tissues and areas of the brain throughout the bloodstream and cerebrospinal fluid. Therefore, melatonin is the message that communicates circadian information throughout the animal, and also signals when the immune function is compromised or varied through seasonal changes. Melatonin acts directly on the immune system through innate and adaptive immune responses in peripheral organs, including the thymus and spleen (Weil et al., 2014). The signaling through melatonin on these organs through the immune system creates a pathway where cytokine signaling from the periphery can inhibit melatonin synthesis. However, short-day lengths and the light from this photoperiod will act on melatonin and enhance the inflammatory response that should normally occur with



The neuroendocrine-loop model of how light acts on the pineal gland to induce melatonin secretion and also peripheral oscillators through a downstream effect (Bell-Pedersen et al., 2015)

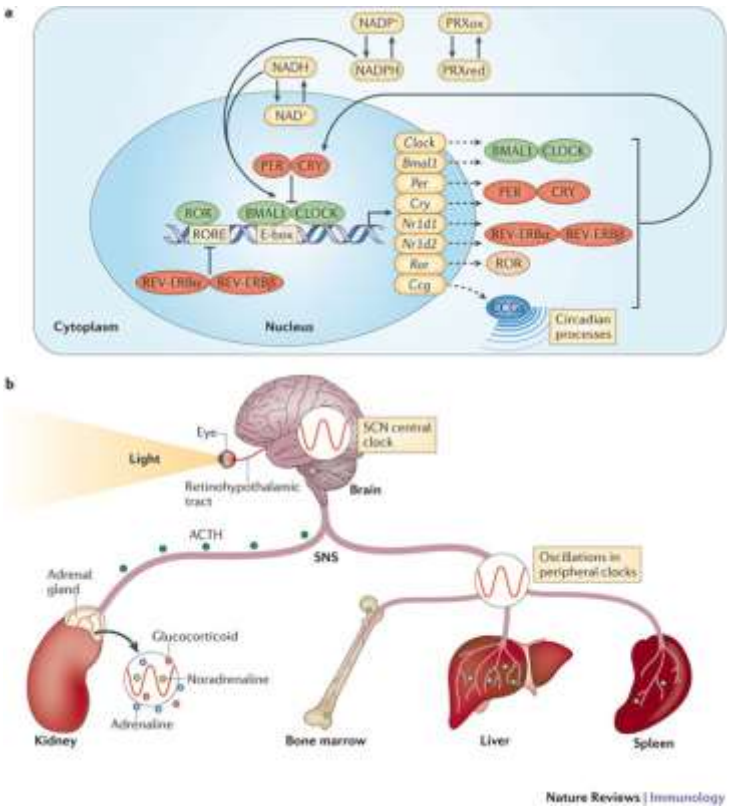
decreasing levels of light (Weil et al., 2014). In other words, animals experiencing short-day lengths will show an increased immune response. This normal response to a winter-like phenotype is easily disrupted. Dim light at night (LAN) blocks enhanced immune function that normally occurs with short-day lengths (Ikeno et al, 2014). According to Ikeno, Weil, and Nelson, LAN has been shown to disrupt short-day response in Siberian hamsters by altering expression patterns of genes implicated in the photoperiodic response (2014). Specifically, LAN changed expression of *Per1*, a circadian clock gene, and also did not indicate signs of decreased gonadal and body mass, or an increased pelage density, that is typically observed with short-day length Siberian hamsters.

The photoperiod not only has a direct influence on melatonin secretion, but also of the genes that are expressed in the periphery and brain from certain lighting cues. Light entrainment of one's daily light–dark cycle occurs from biological, endogenous rhythms, which are heavily connected to seasonal lighting changes. Melatonin secretion, as mentioned above, is directly related to day length. In other words, the duration of nightly melatonin is inversely proportional to day length. However, very low levels of environmental light, such as 1 lux, can suppress pineal melatonin in Siberian hamsters (Weil et al., 2014). Thus, exposure to light at night can disrupt the typical signal for a long-night rhythm and interfere with light-dependent seasonal changes in physiology and behavior (Weil et al., 2014).

In summary, light-dark signals influence the immune system, which is disrupted by exposure to light at night (LAN). Light at night may therefore interfere with immune

function by disrupting the animal's ability to distinguish photoperiod. For instance, short days enhance immune function partially through melatonin signaling, and nightly melatonin secretion is responsible for relaying this day length information. The SCN, or suprachiasmatic nucleus, is located in the hypothalamus and is responsible for controlling neuronal and hormonal activities by interacting with many other parts of the brain. It sends information regarding day length to other hypothalamic nuclei and the pineal gland in order to modulate body temperature and production of hormones such as cortisol and melatonin. The suprachiasmatic nuclei therefore act as a clock and calendar because of their ability to respond to day length information to change the circadian rhythm in animals. In addition, it is important to note that expression of clock genes throughout the SCN encode day length information to coordinate the photoperiod centrally and peripherally. Clock genes have necessary protein products for the maintenance and generation of circadian rhythms throughout all nucleated cells in the organism (Ko and Takahashi, 2006). Since melatonin is responsible for day length information, its secretion determines the amplitude of *Per1*

and/or *Bmal1* expression, which are specific clock genes that then influence other areas of the brain and periphery. Transcriptional-translational feedback loops will then drive the expression of these clock genes (Ko and Takahashi, 2006). For example, in a primary feedback loop, CLOCK and *Bmal1* are positive elements that initiate transcription of target genes (Ko and Takahashi, 2006). These targets contain E-box *cis*-regulatory enhancer sequences, including *Period* (in mice, *Per1, Per2* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*). A negative feedback loop is established from the PER:CRY heterodimers. They will repress their own transcription and work by acting on the CLOCK:BMAL1 complex in the nucleus. Therefore, CLOCK and *Bmal1* are positive elements that drive circadian clockwork through their activation (Ko and Takahashi, 2006). It is also crucial to note that when *Per1* or *Per2* genes are disrupted, arrhythmia in the overall circadian network is a result. Since melatonin secretion is directly proportional to photoperiod, amplitude of *Per1* and an inducible cAMP repressor gene (ICER) in the pars tuberalis (PT) of the pituitary are a good indicator of melatonin activity (Messager et al., 2000). For Siberian hamsters that have long (light: dark (LD) 16:8) and short (SD 8:16) photoperiods, they have a peak of *Per1* and ICER



The transcription of core clock genes and how they act on peripheral organs through oscillations in the peripheral clocks (Scheiermann, Kunisaki, and Frenette, 2013)

gene expression three hours after dawn in the PT (Messager et al., 2000). Their gene expression will serve as a direct representation of photoperiodic time intervals.

We can therefore infer that circadian clocks signals and expression in peripheral immune tissues, such as the lymph nodes and spleen, will regulate inflammatory response rhythms (Bartness et al., 2001). With circadian disruption through light-dark signals, inflammatory responses from these peripheral immune tissues will become deregulated over time. For example, according to Castanon-Cervantes et al., the dysregulation of inflammatory responses through disruption of circadian rhythm are directly proportional to one another (2010). This study concluded that circadian disruption, and not sleep loss or stress, is associated with photoperiodic dysregulation of the innate immune system. This further suggests that peripheral organs involved and those connected to the clock genes are critical for normal circadian function. Specifically, a disrupted immune function may have serious consequences for fitness and/or survival by altering clock genes involved in circadian rhythm. Furthermore, light at night can possibly impact this immune response by changing circadian rhythms. The goal of our study was to study how photoperiod and light at night influence peripheral immune organ clock gene expression and subsequent immune function in Siberian hamsters. Understanding the direct effects of clock gene expression in these organs may lead to methods that prevent heightened immune response in mammals overall.

Methods

Animals. Adult (≥ 60 days of age) male Siberian hamsters (*Phodopus sungorus*) were used in this study. Hamsters were bred under long day (16 hrs light:8 hrs dark) conditions, and then ear-punched and group housed (2-5/cage) in light-proof ventilated cabinets upon group assignment and experiment commencement. Hamsters were randomly assigned to one of four groups in a full factorial design: (1) short day (8:16 (0 lux)), (2) long day (16:8 (0 lux)), (3) short day with dim light at night (8:16 (5 lux)), and (4) long day with dim light at night (16: 8 (5 lux)). Each condition was isolated to an individual cabinet. Daytime light was provided via compact fluorescent bulbs (GE, 12" F8TS Cool White, 8 WATTS), and dim light at night was provided by broad spectrum white LEDs. Illuminance was determined via a light meter at cage level to ensure even distribution of light levels throughout the cabinet. Hamsters were maintained in these conditions for 10 weeks, with weekly body mass measures and cage changes being the only disruptions.

Tissue Collection. At experimental week 10, hamsters were euthanized 'around the clock' at 0500, 0900, 1300, 1700, 2100, and 0100 over the course of two days (all experimental groups were sampled evenly on both days at all time points). Hamsters were individually brought into a procedure room and deeply anesthetized under isoflurane (5%) vapors. Body mass and pelage scores were obtained, and then hamsters were rapidly decapitated and trunk blood was collected. The brain, left inguinal lymph node, and ½ of the spleen were dissected and placed on ice in RNAlater (Qiagen) reagent. The other lymph node and half of

spleen were placed into HBSS (Gibco by Life technologies, calcium, magnesium, 24020) for subsequent culturing. Reproductive organs (epididymides, testes, and seminal vesicles) and gonadal fat pads were dissected and weighed to determine photoperiodic responsiveness.

RNA Extraction and qPCR Analysis. Samples (lymph and spleen) were maintained at 4°C for one week prior to RNA extraction. Lymph node samples (from animals in the same experimental group/time point) were pooled to ensure that adequate RNA was recovered from the samples for cDNA synthesis. RNA was extracted using TRIzol Reagent (Life Technologies, Themo Fisher Scientific Inc.) according to the manufacturer's instructions. RNA pellets were resuspended in 30 µL RNase-free water and quality and quantity were determined using a spectrophotometer (NanoDrop, Thermo Fisher Scientific Inc.). qPCR data used in subsequent analysis were only from samples with RNA yields above 200 ng/µL and with 260/280 and 260/230 ratios between 1.8 and 2.3. RNA was DNAse treated using DNase I (Invitrogen) to prevent amplification of residual genomic DNA. cDNA was synthesized from 0.1 µg RNA using M-MLV reverse transcription (Promega, WI) and diluted 1:10 for subsequent PCR. For real-time PCR analysis, 1% of the cDNA was used at a final concentration of 1x Power SYBR Green PCR Master Mix (Life Technologies) and 0.05 µM of each primer using a 7500 Fast Real-Time PCR System (Life Technologies). Each reaction was performed in duplicate. Forward and reverse primer sequences were 5'-GGT TCG CAG CAG CCA AA-3' and 5'-TGA GGA GTC GAT GCT ACC AAA G-3', respectively, for *Per1*, and 5'-GGC AGC GAT GGC TGT CA-3', and 5'-TCC ACC CAG GCC TGC AT-3', respectively, for *Bmal1* as reported previously (Ikeno & Nelson, 2015). Expression of these targets was normalized to

18s rRNA signal (F and R primer sequences: 18s-1F 5’-GTC TAA GTA CGC ACG GCC GG-3’; 18s-1R 5’-CAT GCA CCA CCA CCC ACG GA-3’) and quantified using a serially diluted standard curve of pooled cDNA.

Results

Light at Night (LAN) Alters the Short-Day Response. The short-day length (winter-like) phenotype showed that it significantly reduced body and reproductive organ masses, as well as alter pelage coloration, to suggest decreased reproductive function. To completely

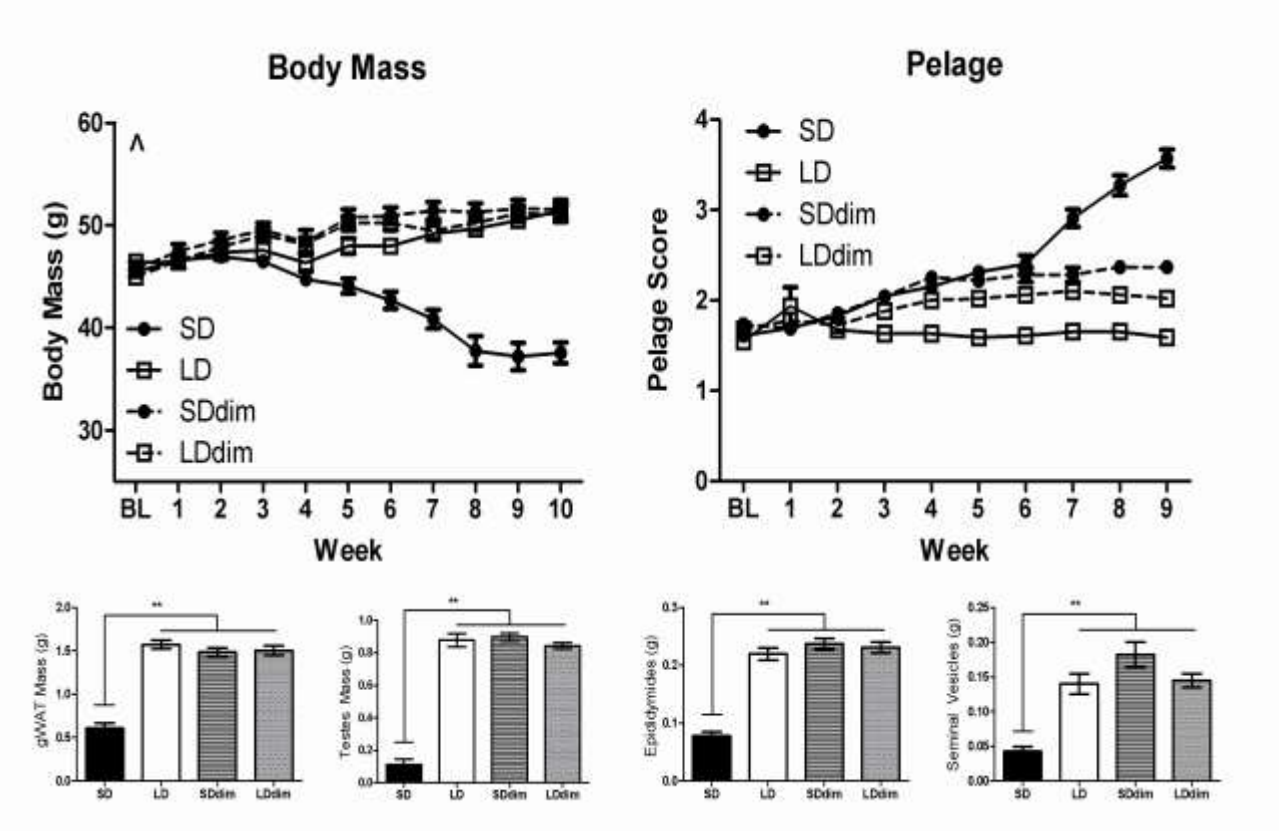


Figure 1. The SD (winter-like) phenotype displayed significantly reduced body and reproductive organ masses, suggesting decreased reproductive function. Additionally, the SD (winter-like hamsters displayed light pelage coloration, which is typical of this phenotype. Light at night interfered with the short-day responses.

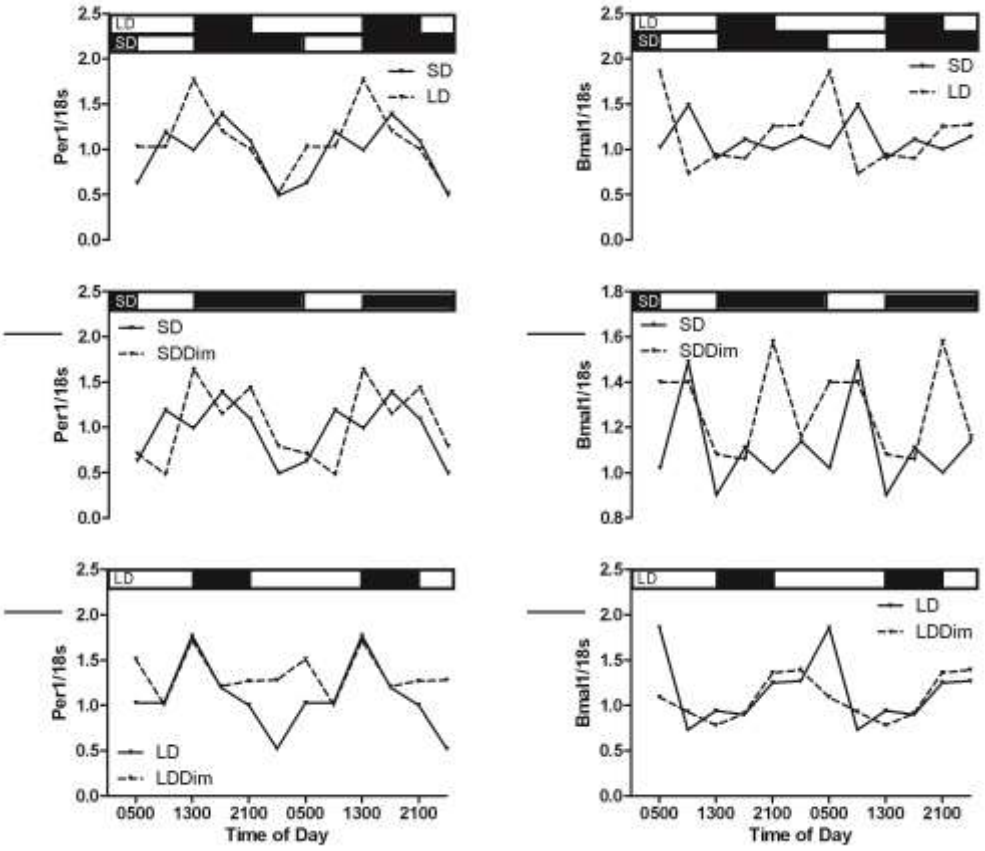
measure the effectiveness of the photoperiod response on the Siberian hamsters, this was also confirmed by comparing the mean tissues masses of reproductive organs in the animals for all the photoperiods. For body mass, it can be observed that the short-day (SD) phenotype greatly decreased in mass over a course of ten weeks. By comparing the SD phenotype to the other three conditions, it is also significant that the SDdim and LDdim phenotypes followed a LD trend of increasing body mass over ten weeks. This is especially important to note for SDdim; with just 5 lux of LAN, this was enough to interfere with the normal short-day response. In addition, when looking at the pelage score, a qualitative measurement of the color of a hamsters' coat, the short-day phenotype is also significantly altered compared to its three counterparts. Over a period of nine weeks, the pelage score for short-day hamsters consistently increased, indicating a white coat, or winter-like, phenotype. If we compare short-day dim to this, it is shown that pelage score also increased, but still followed a trend more consistent with a darker coat, or long-day, phenotype instead. Again, these results suggest that just 5 lux of light at night was enough to alter the normal photoperiodic response for Siberian hamsters.

Reproductive tissue masses also show that light at night interfered with the short-day responses. When comparing the testes, epididymis, and seminal vesicles, significant differences were observed between groups, as well. Short-day length hamsters consistently showed a decreased mass in terms of the reproductive organs, while SDdim hamsters followed the response also shown by LDdim and LD phenotypes. Overall (gonadal white adipose tissue mass [gWAT]) measurements of all the hamsters' shows that short-day

hamsters had a mean mass of just 0.6g while LD, LDdim, SDdim shows mean mass scores of 1.5g. These results demonstrate there was not only an effect of photoperiod on body mass, reproductive organ masses, and pelage score, but also that 5 lux of light at night (LAN) alters the short day response and phenotype.

*Photoperiod and Light at Night Alter Lymph Node Clock Gene Expression***.** After performing qPCR on *Per1* and *Bmal1*, the clock genes involved in circadian function, it can be seen that expression of both genes were asynchronous in the lymph nodes with the added 5 lux of light at night. This suggests that photoperiod also influenced the expression of these clock

Figure 2. Double-plotted values of *Per1* and *Bmal1* gene expression across the circadian day in different photoperiods and lighting conditions.



genes, as well. The left column in Figure 2 demonstrates how *Per1* expression was disrupted by the photoperiod. The first table on the left compares normal SD and LD expression of *Per1* against 18s for all hamsters after ten weeks in the lymph nodes.

At 1300, it is clear that *Per1* is not expressed equally with LD and SD, suggesting that the photoperiod induced more expression in LD Siberian hamsters at this time point and overall in the study. In the second table below in the left column, we are comparing our standard of SD with a SDdim phenotype. It is also clear that at 1300 and now between 500 and 1300, *Per1* expression of SDdim did not follow the same pattern as SD. In other words, just 5 lux of light at night was enough to disrupt this clock gene expression in the lymph node and change the circadian rhythm for all the Siberian hamsters. In the same column, the bottom graph demonstrates a similar trend with LD vs. LDdim expression of *Per1*. However, we see asynchronous expression between 2100 and 0500 for this clock gene.

Bmal1 expression in the right column documents that photoperiod was also influential in disrupting this clock gene. The first table on the right now compares normal SD and LD expression of *Bmal1* against 18s for all hamsters after ten weeks in the lymph nodes. Like *Per1*, we can see that *Bmal1* expression does not operate at the same time of day for LD and SD hamsters, as we would expect. In the table below, we see a very pronounced asynchronous rhythm of *Bmal1* for SD vs. SDdim phenotypes. At multiple time points, this clock gene was not expressed normally compared to the standard SD phenotype. In addition, in the bottom table on the right, although not as robust, suggests the same thing;

photoperiod disrupts this clock gene expression in the lymph nodes and changes the circadian rhythm for all the Siberian hamsters in the study.

Light at Night Alters the Phase Relationship Between Per1 and Bmal1 in Lymph Nodes. The double-plotted values of *Per1* in relation to *Bmal*1 expression show that under different

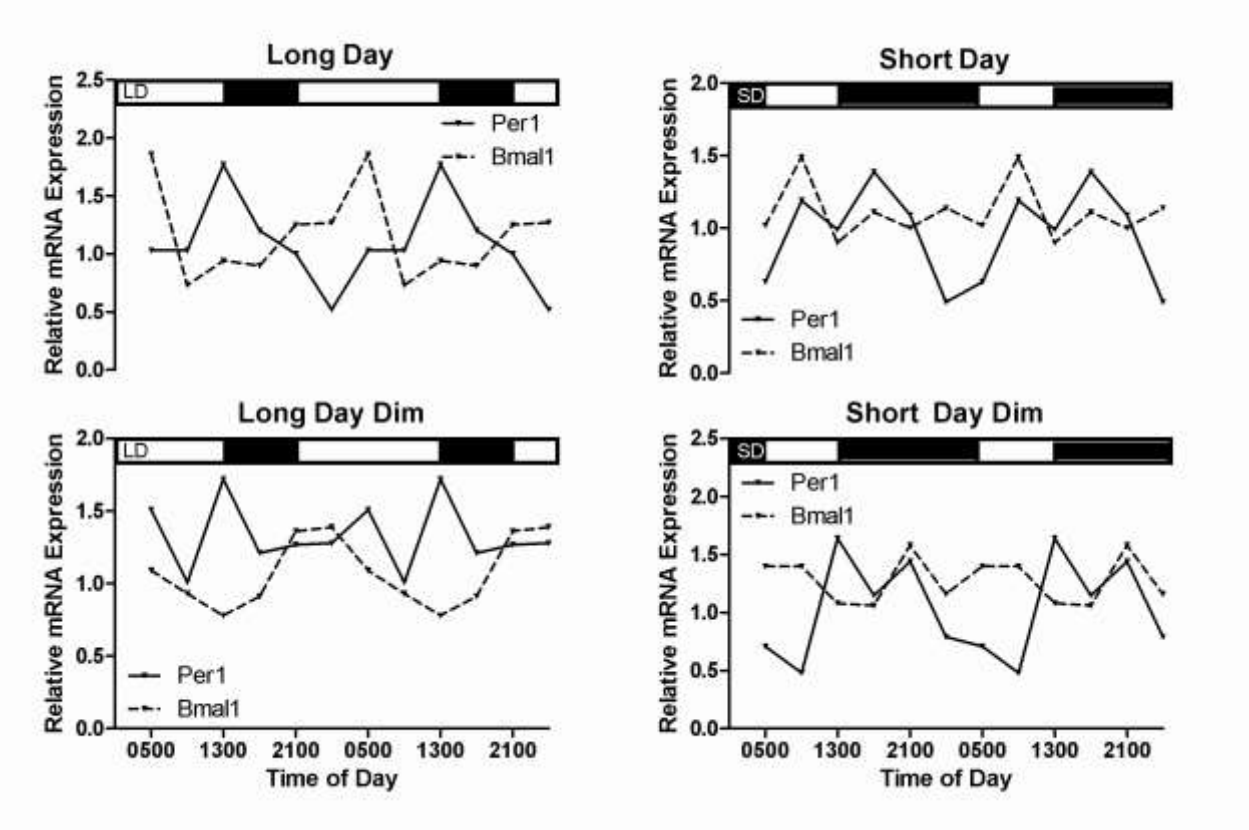


Figure 3. Double-plotted values of *Per1* in relation to Bmal1 expression under different photoperiods and lighting conditions.

photoperiods and lighting conditions, there were clear temporal differences in these clock genes. In the first two tables, it is apparent that with LD and SD phenotypes, *Per1* and *Bmal1* expression differed greatly. For instance, *Per1* vs*. Bmal1* relative mRNA expression for LD hamsters reflected a pattern that was naturally more asynchronous while SD hamsters expressed *Per1* and *Bmal1* more simultaneously during the day. However, when 5

lux of light was added in either LDdim or SDdim conditions, these trends were disrupted greatly. SDdim now follows a pattern more asynchronous relative to its SD counterpart, while LDdim reflected an asynchronous pattern, as well. In other words, light at night altered the temporal profile of both *Per1* and *Bmal1* in each photoperiodic condition to disrupt the overall circadian rhythm for these phenotypes.

Discussion

This study was meant to examine how photoperiod and light at night influence peripheral immune organ clock gene expression and subsequent immune function in Siberian hamsters. It has been shown that light at night may interfere with immune function by disrupting with the animal's natural ability to distinguish photoperiod. The immune system is thought to be directly influenced by the photoperiod and seasonal changes in light patterns, which can be reflected through expression of clock genes involved in circadian rhythm.

Analysis of body and reproductive tissue masses of *P. sungorus*, as well as pelage score, indicate that photoperiod had a direct effect on these values. For example, short-day length hamsters that were exposed to an 8:16 photoperiod had notably less tissue masses overall, as well as increased pelage scores shown through a white coat. In addition, SDdim hamsters under similar conditions (8:16 cycle with 5 lux of light at night) demonstrated that they had disruption of their circadian rhythm with no decrease in body and tissue masses or pelage score. This shows that 5 lux of light at night was enough to impair the reproductive response of these hamsters to short-days. These results, combined with the LDdim and LD

phenotypes that also showed no decrease in tissue masses or a decrease in pelage, confirm the hypothesis that light at night alters the SD response in hamsters, as well as suggest that the immune system may also have been altered in the process.

As noted above, only the lymph nodes were available to analyze for clock gene expression of *Per1* and *Bmal1* due to time constraints. Although results of the qPCR from the spleen were suggesting similar trends to the lymph nodes, data were inconclusive at the time of this presentation. However, the spleen and lymph nodes are interconnected throughout the lymphatic systems in organisms; the spleen has some functions analogous to lymph node function. While the spleen synthesizes antibodies and stores blood platelets, it is also important for normal immune function by fighting bacterial infections (Bender, 2005). The lymph nodes also act as filters for the lymphatic system by fighting infections, and their fluid is eventually sent to the spleen for furthering filtering through the bloodstream. The similarity of these organs allows some preliminary conclusions to be made for the spleen and its effects on the immune system in Siberian hamsters in conjunction with qPCR results from the lymph nodes.

For example, it was clear that photoperiod and light at night altered lymph node clock gene expression of *Per1* and *Bmal1*. Pooled lymph node samples from each group at each time point across 24 hours revealed clear temporal differences in *Per1* and *Bmal1* expression. In addition, there was also clear asynchronous rhythms when comparing SDdim and LDdim against the standards of their respective photoperiods (SD and LD). Furthermore, light at night also altered the phase relationship between *Per1* and *Bmal1* in the lymph

nodes. When 5 lux of light was added in either LDdim or SDdim conditions, expression of *Per1* vs. *Bmal1* varied greatly against the LD or SD phenotypes, again suggesting asynchronous circadian rhythm from disrupted clock gene expression.

Results from this study suggest that through clock gene expression of the spleen and other peripheral organs, such as the lymph nodes, dim light at night impairs the development of the short-day phenotype. However, more analysis of cytokines of these organs is needed to further conclude if the immune system is also directly disrupted with changes in photoperiod. The next step is to determine which cytokine assay is viable for *P. sungorus* by comparing various mice and human cytokine kits. At this time, current results demonstrate that photoperiod and light at night alter lymph node clock gene expression and also that light at night alters the SD response in hamsters.

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